IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of:	Confirmation No. 4915
Ying Luo et al.	Examiner: Janet L. Andres
Application No.: 10/088,961	Technology Center/Art Unit: 1646
Filed: December 30, 2002	<u>DECLARATION UNDER 37 C.F.R § 1.132 OF DR. YASUMICHI HITOSHI</u>
For: TRAF4 ASSOCIATED CELL CYCLE PROTEINS, COMPOSITIONS AND METHODS OF USE	
Customer No.: 20350	

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

I, Yasumichi Hitoshi, M.D., Ph.D., being duly warned that willful false statements and the like are punishable by fine or imprisonment or both (18 U.S.C. § 1001), and may jeopardize the validity of the patent application or any patent issuing thereon, state and declare as follows:

1. All statements herein made of my own knowledge are true, and statements made on information or belief are believed to be true and correct.

2. I received my medical degree from Kumamoto University Medical School in 1987. I received a Ph.D. in immunology from The Institute for Medical Immunology, Kumamoto University Medical School in 1991. I was a postgraduate research associate at the Institute for Medical Immunology, Kumamoto University Medical School in 1991 and at the Institute of Medical Science, The University of Tokyo from 1992-1995. From 1995-1998 I was a postdoctoral fellow in the Department of Molecular Pharmacology at Stanford University. A copy of my curriculum vitae is attached hereto as Exhibit D.

<u>EXHIBIT A</u>

3. I have worked in the department of Cell Biology at Rigel Pharmaceuticals, Inc. since 1998. Currently, I am Director and Project Leader at Rigel Pharmaceuticals, Inc.

4. The present invention claims Mkinase protein, a cell cycle protein that binds to the TRAF4 protein.

5. I have read and am familiar with the contents of the patent application. In addition, I have read the Office Action, mailed June 9, 2004, received in the present case. It is my understanding that the Examiner believes that the present invention does not provide a "specific and substantial" use for the claimed nucleic acids. This declaration is provided to demonstrate that the Mkinase cell cycle protein has "specific and substantial" utility based on the specification and the utility of a closely related NTKL protein.

6. The Mkinase protein was identified by Scientists at Rigel Pharmaceuticals in a two hybrid screen using the TRAF4 protein as "bait." Thus, Mkinase binds to the TRAF4 protein. The TRAF4 protein is overexpressed in some cancer cells and is recognized by those of skill to play a role in tumorigenesis by regulating signal transduction pathways. It is my opinion that those of skill would believe that the Mkinase protein has a role in tumorigenesis and signal transduction based on its ability to bind to the TRAF4 protein.

7. The Mkinase protein is closely related to the NTKL protein. I have read a reference by Kato *et al.* that describes identification and characterization of the NTKL protein. (Kato *et al*, *Genomics* 79:760-767 (2002), enclosed as Exhibit B.) According to Kato *et al.*, the NTKL protein contains a conserved kinase domain and maps to a breakpoint region on chromosome 11 that is associated with cancer. I have also compared the sequence of the Mkinase protein to the sequence of the NTKL protein and the sequence alignment is submitted as Exhibit C. The alignment indicates that the NTKL sequence and the Mkinase sequence share 99% identity. Based on Kato *et al.* and comparison of the NTKL and Mkinase amino acid sequences, I believe it more likely than not that the Mkinase gene is useful as a diagnostic for cancer.

8. At the time of filing, the kinase domain of the Mkinase protein was identified by the Applicants using well-known sequence comparison programs. *See, e.g.*, specification at page 4, lines 13-15; page 6, line 26 through page 7, line 27; page 56, lines 16-20; and Figure 7A and 7B. Also at the time of filing, Applicants disclosed that the Mkinase protein binds to the TRAF4 protein, which was recognized to have a role in tumorigenesis. In my opinion, the Kato *et al.* reference described above confirms the identification of the Mkinase protein as a protein or gene product associated with cancer, as asserted in the specification. Also in my opinion, the discovery of the Mkinase associated kinase activity and the binding of Mkinase to the TRAF4 protein would lead those of skill to believe that the Mkinase protein was a protein involved in tumorigenesis.

9. In view of the foregoing, it is my scientific opinion that one of skill in the art, at the time the application was filed, would recognize the utility of the Mkinase proteins of the present invention.

Date: 12/7/04 By: [signature]

Yasumichi Hitoshi, M.D., Ph.D.

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Identification and Characterization of the Human Protein Kinase-like Gene *NTKL*: Mitosis-Specific Centrosomal Localization of an Alternatively Spliced Isoform

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Although the centrosome has an essential role in mitosis, its molecular components have not been fully elucidated. Here, we describe the molecular cloning and characterization of the human gene *NTKL*, which encodes an evolutionarily conserved kinase-like protein. *NTKL* mRNA is found ubiquitously in human tissues. *NTKL* is located on 11q13 and is mapped around chromosomal breakpoints found in several carcinomas, suggesting that *NTKL* dysfunction may be involved in carcinogenesis. Alternative splicing generates two variant forms of *NTKL* mRNA that encode protein isoforms with internal deletions. When fused to green fluorescent protein, the full-length product and one of the variant proteins are found in cytoplasm. The other variant product also exists in the cytoplasm during interphase, but is found in the centrosomes during mitosis. Endogenous NTKL protein is also localized to the centrosomes during mitosis. This cell-cycle-dependent centrosomal localization suggests that *NTKL* is involved in centrosome-related cellular functions.

Key Words: *NTKL, Homo sapiens*, protein kinase, alternative splicing, gene family, centrosome, mitosis, multimer formation

Introduction

Mitotic cell division requires tight control of spindle formation and the subsequent segregation of condensed chromosomes to ensure faithful transmission of entire genomes to daughter cells. The centrosome, composed of a pair of centrioles and associated electron-dense pericentriolar materials, has an essential role in this process. The centrosome lies close to the nucleus at interphase and undergoes duplication that is linked to the onset of DNA replication at the G1/S transition. At the end of the G2 phase, the duplicated centrosomes migrate to opposite sides of the nucleus. At mitosis, cytoplasmic microtubules are organized into bipolar spindles. The centrosome functions as a microtubule-organizing center and has a critical role in accurate chromosome segregation [1,2].

Defects in centrosome function lead to aberrant chromosomal segregation and changes in chromosome numbers and structure [3]. These chromosomal abnormalities are well recognized as the predominant class of genetic instability found in cancer cells [4] and are also believed to promote the abnormal growth and metastasis of cancer cells [5]. Indeed, various human carcinomas exhibit hypertrophy of the centrosomes [6–8]. Therefore, it is believed that the determination of centrosome function at the molecular level will contribute to the understanding of how carcinomas develop.

The importance of the centrosome has been recognized, but its molecular components have not been fully characterized. The γ-tubulin protein is a major component of the centriole core and is essential for microtubule nucleation [9]. The centrosome is also associated with regulatory factors, such as TP53 [10], RB1 [11], CCNB1 [12], CDC2 [13], and BRCA1 [14], in cell-cycle-dependent manners. Increasing numbers of protein kinases have been reported to be located at the centrosome [15]. These include aurora-related kinases required for centrosome separation and mitotic spindle assembly [16], polo-like kinases involved in centrosome maturation and bipolar spindle formation [17], and NIMA-related kinases that regulate centrosome separation [15]. Some of these genes are overexpressed in human carcinomas [18–21], suggesting the possible involvement of abnormal regulation of centrosomal kinases in carcinogenesis and tumor progression.

Here, we describe the molecular cloning and characterization of *NTKL*, which encodes a protein with similarity to protein kinases at its amino-terminal region. Searching DNA databases revealed that *NTKL* is a member of a large family found in a broad range of eukaryotes. We identified three

EXHIBIT B

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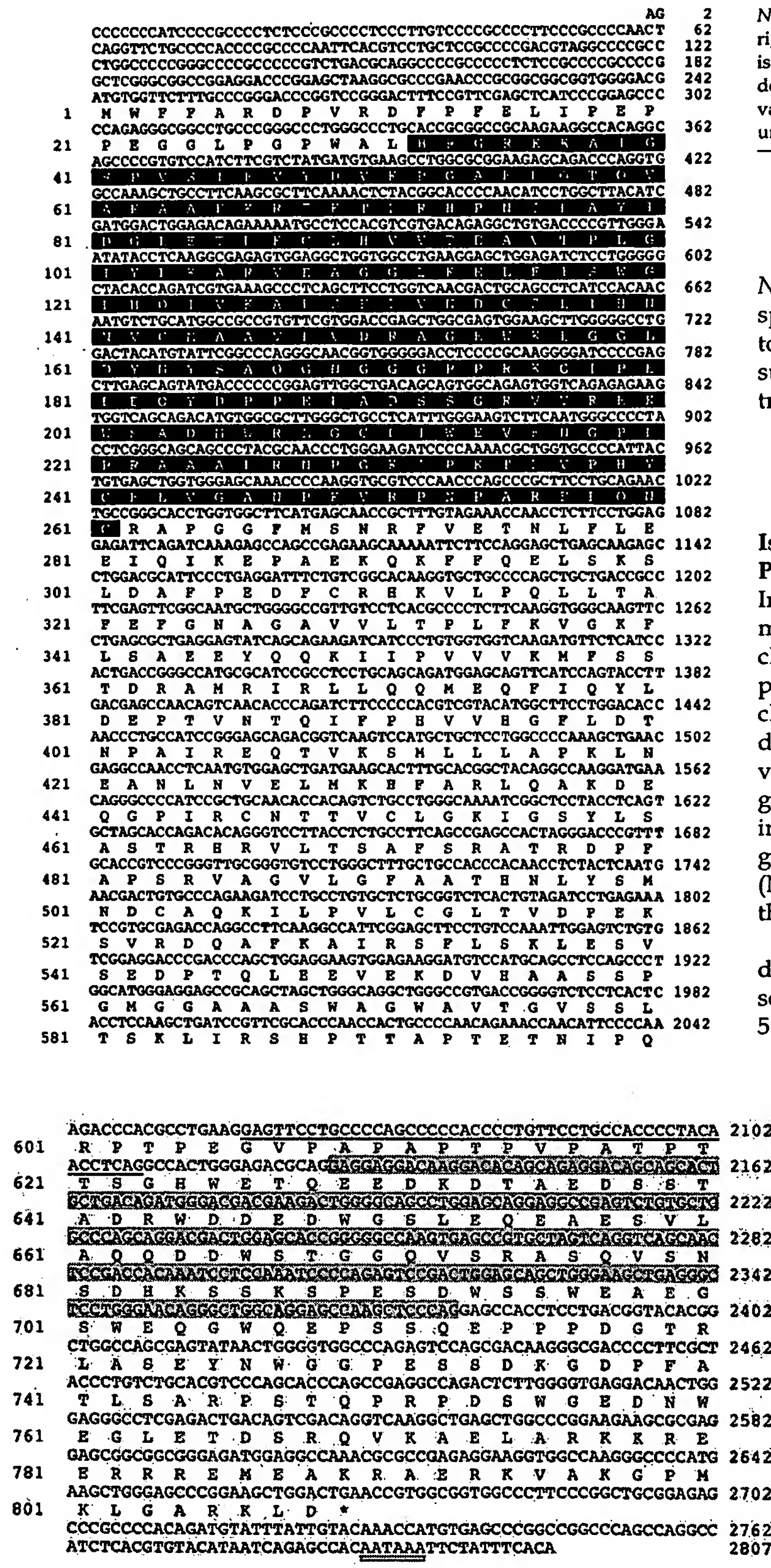

FIG. 1. Nucleotide and deduced amino acid sequences of human *NTKL* cDNA. Nucleotides and amino acids are numbered at the right and left sides, respectively. The protein kinase-like domain is shaded in black. The underlined area indicates the region deleted in both variant 1 and variant 2. The region spliced out in variant 2 is shown in gray. A possible poly(A) signal is double-underlined.

NTKL cDNA isoforms generated by alternative splicing. One of the isoform proteins was localized to the centrosomes during mitosis. Our observations suggest the probable involvement of *NTKL* in centrosome functions.

RESULTS

Isolation, Sequence Analysis, and Expression Profile of *NTKL*

In the course of large-scale sequencing of a human mammary gland cDNA library, we isolated cDNA clones that seemed to encode a novel kinase-like protein. We also isolated two additional cDNA clones with internal deletions in the 3′ regions. We designated these clones as full-length, variant 1, and variant 2, respectively. (We first named this novel gene *GKLP* and deposited the nucleotide sequences in DDBJ/EMBL/GenBank, but later changed the gene name from *GKLP* to *NTKL* (N-terminal kinase-like) on a recommendation by the HUGO Gene Nomenclature Committee.)

To obtain the entire open reading frame, we designed nested primers from the nucleotide sequence of the *NTKL* cDNA clone and carried out 5′-RACE using mammary gland cDNA. Two successive rounds of PCR using the nested primers yielded an 800-bp cDNA fragment. Sequence analyses of the cDNA clones and the cDNA fragment isolated by 5′-RACE resulted in the assembly of a single, large, open reading frame (Fig. 1). This open reading frame started from a strong consensus initiation sequence and encoded a putative protein of 808 amino acids with a predicted molecular mass of 89.6 kDa. The putative protein encoded by the variant 1 cDNA consisted of 791 amino acids and lacked the region corresponding to amino acids 606–622 of the full-length *NTKL*. Variant 2 consisted of 707 amino acids and lacked two corresponding regions of the full-length *NTKL* (amino acids 606–622 and 629–712). Analysis of the deduced primary sequence using the Conserved Domain program suggested that the NTKL protein contains a

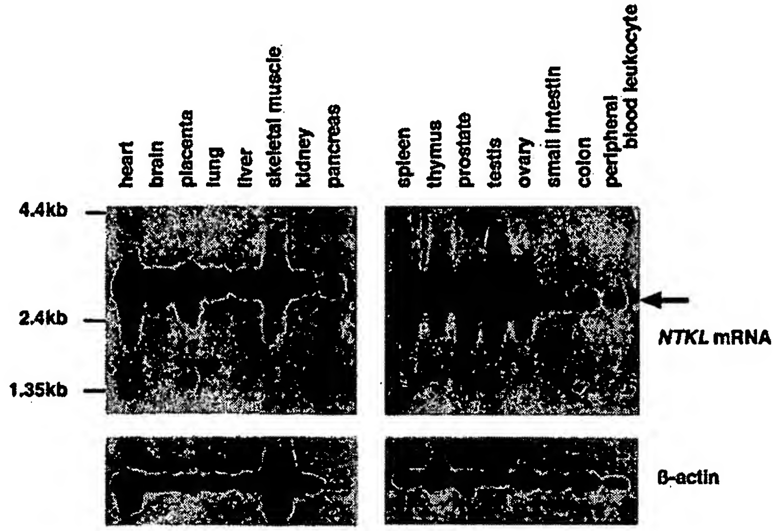

FIG. 2. Tissue distribution of *NTKL* mRNA. NTKL cDNA was labeled with [α-^{32}P]dCTP by random priming and hybridized to a multiple tissue northern blot membrane. In the lower panel, expression of β-actin mRNA is shown as a loading control.

kinase-like domain in the N-terminal region (amino acids 32–261). A cluster of basic amino acid residues was found in the carboxy-terminal region. No other protein motifs were observed in the deduced primary structure.

We then examined the tissue distribution of *NTKL* mRNA. We carried out northern blot analysis using a multiple-tissue blot membrane and detected 2.8-kb *NTKL* mRNA in all tissues (Fig. 2).

Evolutionary Conservation of *NTKL*

Searching the GenBank database with NTKL sequence revealed several proteins with significant homology to human NTKL (Fig. 3). Human NTKL is most closely related to the mouse NTKL homolog (90% identity at the amino acid level), which was originally isolated as a factor interacting with protein kinase B [22]. Proteins structurally similar to human NTKL were found in *Drosophila melanogaster*, *Caenorhabditis elegans*, and *Arabidopsis thaliana*. These proteins carry protein kinase-like domains in the N-terminal regions. YOR112W of *Saccharomyces cerevisiae* is predicted to encode an NTKL-related protein that shows no significant similarity to protein kinases.

Genomic Structure and Alternative Splicing of *NTKL*

Searching by BLAST, we found that the nucleotide sequence of the *NTKL* cDNA matched two human genome draft sequences (GenBank acc. nos. AF255613 and Hs11_25998). To investigate the genomic organization of *NTKL*, we compared the cDNA and genomic sequences. *NTKL* was mapped to chromosome 11q13 and spanned approximately 15 kb between microsatellite markers *D11S4933* and *D11S546*. This genomic region is known to contain breakpoints for chromosomal translocations reported in two cases of extragonadal germ cell tumors and in one case of renal cell carcinoma [23–25]. A detailed sequence analysis of the genomic region between *D11S4933* and *D11S546* on 11p13 has been reported [26]. *NTKL* was located in the genomic region where the breakpoints are suspected to exist [26] (data not shown), although the precise position of the chromosomal breakpoints is yet to be determined.

NTKL is composed of 18 exons (Fig. 4A). As described above, we isolated two C-terminal variants that seemed to be generated by alternative splicing. Variant 1 lacks the 5′-half of exon 14. Variant 2 uses different splice acceptor and donor sites in exon 14, skips over the entire exon 15, and lacks the 5′ half of exon 16 (Fig. 4B). As the translational reading frames of these splicing variants are the same as the full-length *NTKL* mRNA, the two variant mRNAs encode proteins with internal deletions (Fig. 1).

Biochemical Characterization of NTKL

To investigate the function of NTKL, we searched for proteins that interact with NTKL by yeast two-hybrid screening of a human mammary gland cDNA library. We isolated several positive clones that were then found to be also *NTKL* (data not shown). To examine the multimer formation of NTKL in mammalian cells, we coexpressed FLAG- and hemagglutinin epitope (HA)-tagged NTKL proteins in COS7 cells and performed immunoprecipitation with an anti-FLAG antibody. HA-tagged NTKL coprecipitated with FLAG–NTKL, confirming the multimer formation of NTKL *in vivo* (Fig. 5A).

To examine the state of the endogenous NTKL protein, we raised an antibody against NTKL. The antibody showed highly specific recognition of the endogenous NTKL protein in western blot analysis (Fig. 5B). The mobility of the endogenous NTKL protein was slower than the calculated molecular weight. The FLAG-tagged NTKL protein, which was expressed from the plasmid, also showed slower mobility (data not shown), suggesting that NTKL protein is modified posttranslationally. We then examined the multimer formation of the endogenous NTKL protein. Whole-cell extract was prepared from MCF7 and reacted with bis(sulfosuccinimidyl)suberate (BS3), a bipolar cross-linking reagent. The monomeric form of the NTKL protein was converted to the larger species (approximately 300 kDa) in a BS3 concentration-dependent manner (Fig. 5C). Similar results were obtained with another cross-linking reagent, disuccinimidyl suberate (data not shown). These observations were consistent with the complex formation of the exogenously expressed NTKL proteins in COS7 and the NTKL–NTKL interaction in yeast two-hybrid assays. We therefore concluded that the NTKL protein, at least in part, forms a multimer, most likely a trimer.

Next, we examined whether NTKL possessed protein kinase activity based on its sequence similarity to protein kinases. However, immunoprecipitated FLAG-tagged NTKL

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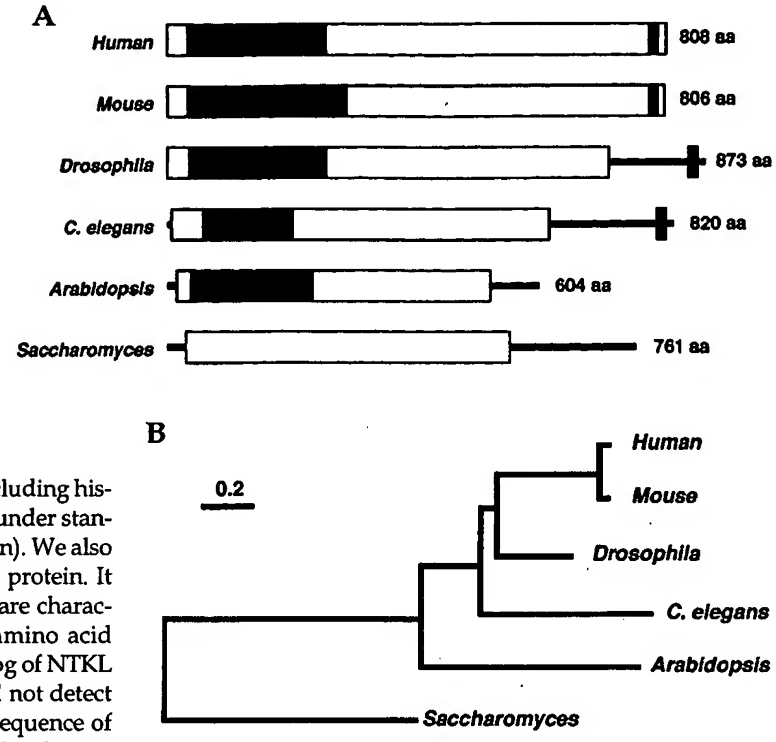

FIG. 3. Evolutionary conservation of *NTKL*. (A) Schematic representation of NTKL-related proteins. BLAST search using the human *NTKL* cDNA sequence against the GenBank database revealed the existence of open reading frames that are predicted to encode NTKL-related proteins in the following eukaryotic species: YOR112 of *S. cerevisiae* (GenBank acc. no. CAA99310), At2g40730 of *A. thaliana* (AC007660), W07G4.3 of *C. elegans* (CAB01444), and CG1973 of *Drosophila* (AAF56933). Murine *NTKL* homolog (AF276514) was also detected. Structures of human NTKL and related proteins are shown. Boxes indicate regions of similarity to the human NTKL. Regions with similarity to protein kinases are shown in black. Gray boxes show clusters of basic amino acids. Thick bars indicate regions that have no similarity to human NTKL protein. (B) Phylogenic tree of NTKL-related proteins. The phylogenic relationships of the NTKL-related proteins were analyzed using ClustalW. The phylogenic tree was depicted using the DendroMaker program.

protein did not phosphorylate purified proteins, including histone H1, enolase, myelin basic protein, and casein, under standard *in vitro* kinase assay conditions (data not shown). We also failed to detect autophosphorylation of the NTKL protein. It has been proposed that almost all protein kinases are characterized by 12 subdomains, in which important amino acid residues are well conserved [27]. The mouse homolog of NTKL lacks one subdomain (subdomain I) [22]. We could not detect this subdomain motif in the deduced amino acid sequence of human NTKL. Together with the results of *in vitro* kinase assays, we concluded that while human NTKL exhibits a protein kinase-like structure, it does not possess kinase activity.

Centrosomal Localization of the NTKL Variant 2–EGFP and Endogenous NTKL during Mitosis

To identify a possible site of action for the NTKL protein and to obtain information about its function, we examined the subcellular localization of NTKL during the cell cycle. We constructed plasmids that expressed each NTKL isoform as a fusion to enhanced green fluorescent protein (EGFP). The plasmids were introduced into MCF7 cells. The subcellular localization of the fusion proteins was observed by fluorescence microscopy. Full-length and variant 1 NTKL–EGFP fusion proteins were localized to the cytoplasm throughout the cell cycle (data not shown). However, the variant 2–EGFP fusion protein exhibited mitosis-specific localization to centrosomes (Fig. 6). During interphase, variant 2–EGFP was also found in cytoplasm, similar to full-length and variant 1 products (data not shown). At the beginning of mitosis, a pair of bright green foci was observed in the nucleus that emerged as the chromosomes became condensed (Figs. 6A–6D). At prometaphase, the foci migrated to opposite poles of the nucleus (Figs. 6E and 6F). We also stained the transfected cells with an anti-α-tubulin antibody and found that the foci of the variant 2–EGFP fluorescence were localized to the spindle poles. During chromosomal segregation, variant 2–EGFP comigrated with the spindle poles (Figs. 6G–6P). In addition to the punctate staining at the

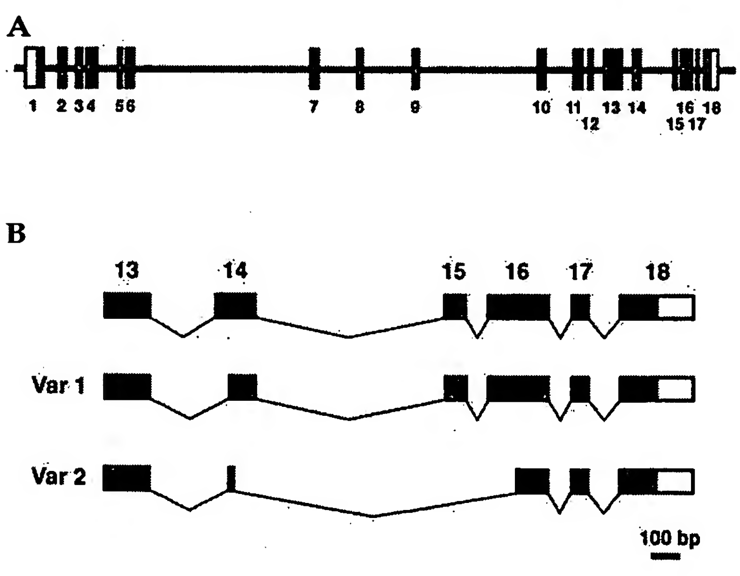

FIG. 4. Genomic organization of *NTKL*. (A) Structure of the human *NTKL* gene. Exons are numbered and shown as boxes. Protein-coding regions are shaded. The 5′- and 3′-untranslated regions are shown by open boxes. (B) Alternative splicing of *NTKL*. Exons 13–18 are highlighted. Full-length *NTKL* mRNA comprises 18 exons. Splicing variant 1 lacks the 5′ half of exon 14. Splicing variant 2 uses different splice donor and acceptor sites in exon 14, and skips over exon 15 and the 5′-half of exon 16. Nucleotide sequences skipped in the splicing variants are shown in Fig. 1.

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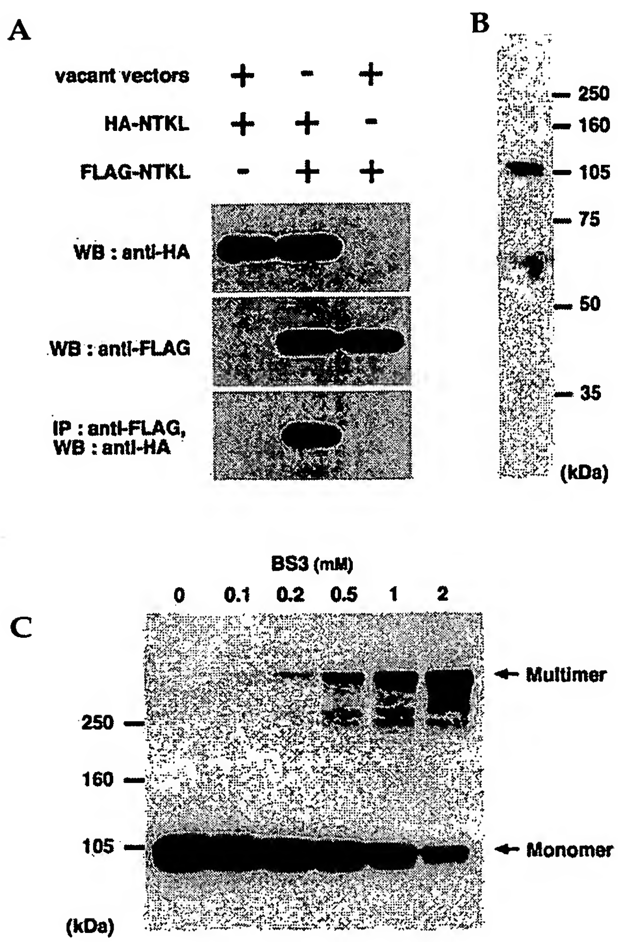

FIG. 5. Multimer formation of the NTKL protein. (A) Complex formation exogenously expressed FLAG- and HA-tagged NTKL proteins. COS7 cells were cotransfected with combinations of pFLAG-NTKL, pHA-NTKL, and vacant vectors as indicated at the top of the panels. Immunoprecipitation was carried out with anti-FLAG monoclonal antibody. Complex formation of the FLAG-tagged and HA-tagged NTKL proteins was analyzed by western blot. (B) Detection of endogenous NTKL protein by western blot. Whole-cell extract of MCF7 was subjected to SDS-PAGE followed by western blot with the anti-NTKL antibody. (C) Cross-link analysis of the endogenous NTKL protein. A bipolar cross-linking reagent BS3 was reacted with the whole-cell extract of MCF7 at the indicated concentrations. Multimer formation was analyzed by SDS-PAGE followed by western blot with the anti-NTKL antibody.

centrosomes, diffuse cytoplasmic fluorescence was also observed throughout mitosis. The cells transfected with EGFP alone showed no fluorescent foci at the centrosomes (data not shown). In the presence of nocodazole, the centrosomal localization was observed (Figs. 6Q–6R), suggesting microtubule polymerization is not required for the centrosomal localization of the variant 2–EGFP. Similar microtubule-independence was reported in several centrosomal proteins [28–30].

Next, we examined the subcellular localization of endogenous NTKL. The MCF7 cells were fixed in methanol/acetone mixture and stained with the anti-NTKL antibody. In this fixation condition, the signals for cytoplasmic NTKL protein were reduced, probably because soluble cytoplasmic NTKL protein was washed out. The endogenous NTKL protein was concentrated around the centrosomes at the mitotic stage (Figs. 6U and 6V). Finally, we performed the immunostaining with another cell line, HeLa, and observed similar centrosomal staining of the endogenous NTKL protein (Figs. 6W and 6X). These observations indicated that a fraction of endogenous NTKL is concentrated to the centrosomes during mitosis.

Discussion

During the large-scale sequencing of human mammary gland cDNA, we isolated *NTKL* cDNA clones and two *NTKL* cDNA variants generated by alternative splicing. *NTKL* encoded a protein with sequence similarity to protein kinases in the N-terminal region. Searching the GenBank database, we found that several genes from a broad range of eukaryotes encoded proteins structurally related to human NTKL. To obtain insights into *NTKL* function, we examined the subcellular localization of the NTKL isoforms. While full-length and variant 1 NTKL proteins fused to EGFP were found in the cytoplasm throughout the cell cycle, NTKL variant 2–EGFP was concentrated to the centrosomes during mitosis in addition to being present in the cytoplasm during interphase. By using the anti-NTKL antibody, we observed that a fraction of endogenous NTKL was concentrated to the centrosomes during mitosis.

Although the primary structure of the NTKL protein showed significant similarity to protein kinases, immunoprecipitated FLAG-tagged NTKL protein did not exhibit kinase activity, despite the use of several purified proteins as substrates for the *in vitro* kinase assays. A subdomain shared by almost all protein kinases was absent from the human NTKL protein. As some NTKL-related proteins from other species also lack this subdomain motif, it is possible that human *NTKL* and the other related genes may derive from a common ancestral gene that once encoded a protein kinase but then evolved to lose kinase activity. A prime example of such differential evolution from an ancestral gene is *GAL1* and *GAL3* of *Saccharomyces cerevisiae*. The high similarity at the amino acid level suggests that *GAL1* and *GAL3* evolved from a single ancestral gene. However, Gal1 protein functions as a galactokinase that phosphorylates galactose, whereas Gal3 protein has no kinase activity but functions as a galactose sensor [31,32]. It is thought that the Gal3 protein first gained a novel function as a galactose sensor and then lost its kinase activity during evolution. Thus, it is possible that *NTKL* family members may have gained some new function and then lost their protein kinase activities. It is also possible that NTKL might have some protein kinase-related function, such as recognition and binding of phosphorylated proteins.

Another example for kinase-independent functions was reported in kinase suppressor of Ras (KSR). KSR shares

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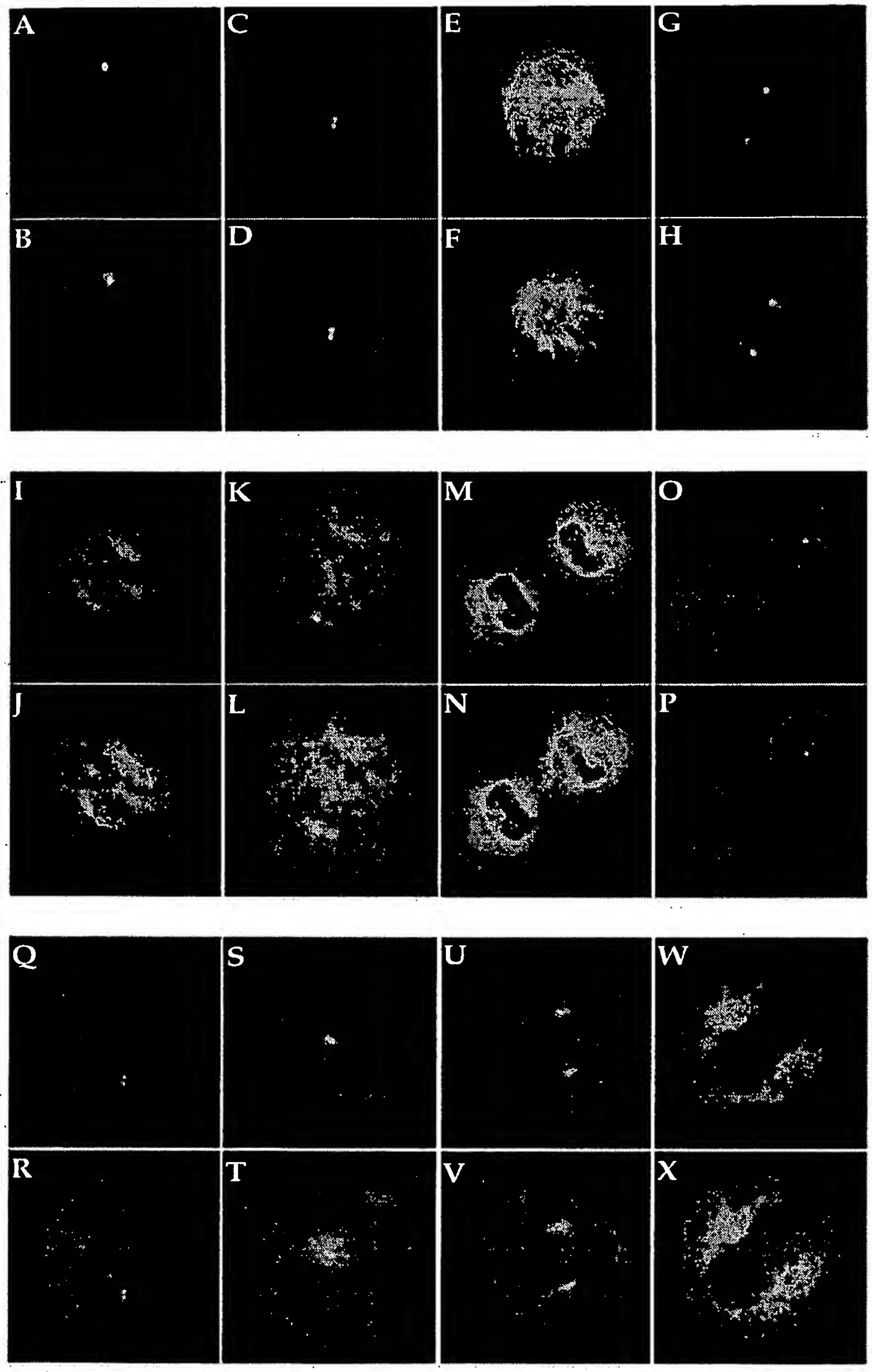

FIG. 6. Subcellular localization of NTKL variant 2-EGFP and endogenous NTKL. (A–P) Subcellular localization of NTKL variant 2-EGFP in MCF7 at mitosis. Cells were transfected with the NTKL variant 2-EGFP expression plasmid and treated with nocodazole at 150 ng/ml for 10 hours to increase the fraction of mitotic cells. Following washes with phosphate-buffered saline, mitosis was allowed to proceed for 75 minutes. Cells were then stained with anti-γ-tubulin antibody (red) and DAPI (blue). Subcellular localization of NTKL variant 2-EGFP was observed by fluorescence microscopy (A, C, E, G, I, K, M, and O). Images of α-tubulin staining were merged with those of NTKL variant 2-EGFP (B, D, F, H, J, L, N, and P). Stages of cell division are as follows: (A–D), prophase; (E) and (F), prometaphase; (G) and (H), metaphase; (I–L), anaphase; (M–P), telophase. (Q–T) Microtubule-independent centrosomal localization of the NTKL variant 2-EGFP. Subcellular localization of the NTKL variant 2-EGFP was observed in the presence (Q and R) or absence (S and T) of nocodazole (150 ng/ml) as above. (U–X) Centrosomal localization of the endogenous NTKL protein during mitosis. MCF7 (U and V) and HeLa (W and X) were double-stained with anti-NTKL (green) and anti-α-tubulin (red) antibodies. Nuclei were shown by staining with DAPI (blue).

sequence homology with Raf family kinases and has an important role in Ras-mediated signal transduction. KSR interacts with several components of the MAP kinase cascade to form a large protein complex. Similar to NTKL, KSR lacks several key properties of known protein kinases and functions in a kinase-activity-independent manner [33,34].

In this study, we identified three NTKL isoforms apparently derived by alternative splicing. Alternative splicing is an important means of generating protein diversity in eukaryotes. An increasing number of examples have been reported that alternative splicing can yield isoforms with different subcellular localization. For example, the nuclear mitotic apparatus protein NuMA1 is composed of three isoforms derived by alternative splicing, and these isoforms are differentially localized to the nucleus and centrosome [35]. Differential localization of alternative splicing-derived isoforms in the nucleus and cytoplasm has also been reported for a number of other proteins, including FGF3 [36], DNTT [37], NF2 [38], and BACH1 [39]. In *BCL2L1*, alternative splicing generates long and short transcripts that encode proteins with positive and negative effects on apoptosis, respectively [40]. In the case of NTKL, the full-length and variant 1 products are present in the cytoplasm throughout the cell cycle, whereas variant 2 product is concentrated to centrosomes during mitosis. The differential subcellular localization of the NTKL

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isoforms might reflect functional differences among the isoforms. It is also an interesting issue whether the alternative splicing of *NTKL* is regulated in cell-cycle-, developmental-stage-, or tissue-dependent manners.

The evolutionary conservation of *NTKL* among a wide range of eukaryotic species raises the possibility that *NTKL* might have a fundamental role in a cellular function common to eukaryotic cells. This speculation is supported by the observation that the expression of *NTKL* mRNA was virtually ubiquitous in all human tissues tested. In this respect, it is noteworthy that the variant 2 protein was concentrated to centrosomes at mitosis, which suggests that the NTKL variant 2 protein may have a mitosis-related function, such as in spindle formation or segregation of condensed chromosomes. Recently, Liu *et al.* showed by cell fractionation that mouse NTKL protein was concentrated in the low-density microsomal fraction [22]. While this fraction contained Golgi apparatus, cytoskeletons, and other small cellular compartments, it was still unclear where mouse NTKL protein was localized during mitosis. While the differential subcellular localization of the NTKL isoforms might reflect specific cellular roles, the precise function of NTKL remains to be elucidated. The significance of NTKL multimer formation and the possible involvement of dysfunction of *NTKL* in carcinogenesis are also yet to be investigated. Further research on human *NTKL*, together with the analysis of *NTKL*-related genes in model organisms, is required to address these questions.

Materials and Methods

Cloning of NTKL cDNA. *NTKL* cDNA clones were isolated during the large-scale sequencing of cDNA clones from a Matchmaker human mammary gland cDNA library (Clontech, Palo Alto, CA). To obtain the 5′-end of the *NTKL* cDNA, 5′-RACE was carried out using Marathon-Ready human mammary gland cDNA (Clontech) according to the manufacturer's protocols. Primers used for 5′-RACE were R1, 5′-GGGGTCATACTGCTCAAGCT-3′, and R2, 5′-TGCCACTGCTGTCAGCCAAC-3′. The 5′-RACE yielded a 0.8-kb cDNA fragment, which was subcloned into the pT7Blue plasmid vector (Novagen, Madison, WI). Nucleotide sequences of the isolated cDNAs were determined using a CEQ2000XL DNA analysis system (Beckman Coulter, Fullerton, CA).

Computer analysis of NTKL sequence. Homology search was performed by BLAST using default parameters (http://www.ncbi.nlm.nih.gov/BLAST/). Exon–intron boundaries were determined by comparison of *NTKL* cDNA nucleotide sequences to human draft sequences (GenBank acc. no. AF255613 and Hs11_25998). Protein motifs in the deduced amino acid sequence were predicted by searching the Conserved Domain Database with reverse position-specific BLAST (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi). Phylogenic relationships among human NTKL and related proteins were assessed using the ClustalW program at DDBJ (http://www.ddbj.nig.ac.jp/E-mail/clustalw-e.html) and shown as a phylogenic tree by DendroMaker (http://www.cib.nig.ac.jp/dda/timanish/dendromaker/home.html).

Plasmid constructs. *NTKL* cDNA clones isolated from the human mammary gland cDNA library were ligated in-frame with the cDNA fragment obtained by 5′-RACE to generate entire cDNAs of the isoforms. The entire cDNA of each isoform was subcloned into the pEGFP-C (Clontech), pFLAG-CMV2 (Sigma, St. Louis, MO), and pcDNAHA plasmids, respectively. pcDNAHA was constructed by replacing the Xpress epitope of the pcDNA 3.1His (Invitrogen, Carlsbad, CA) with an HA epitope. All plasmids constructed in this study were verified by sequencing as described above.

Northern hybridization. *NTKL* cDNA was labeled with [α-^{32}P]dCTP by random priming using the Megaprime DNA labeling system (Amersham Pharmacia Biotech, Piscataway, NJ) according to the manufacturer's protocols. The probe was hybridized to a human multiple tissue northern membrane (Clontech) according to the manufacturer's protocols. Signals for *NTKL* mRNA were detected by autoradiography. The membrane was reprobed with ^{32}P-labeled β-actin cDNA to show equivalent loading of poly(A)$^+$ RNA in each lane.

Antibody production. A cDNA fragment corresponding to the amino acids 484–707 of *NTKL* variant 2 was subcloned in-frame into pGEX plasmid (Amersham Pharmacia Biotech). A glutathione S-transferase (GST)-NTKL fusion protein was expressed in *Escherichia coli* BL21(DE3) and purified with glutathione-agarose beads (Amersham Pharmacia Biotech). Anti-NTKL antibody was raised by immunizing rabbits with the GST-NTKL protein (Takara Shuzo, Ohtsu, Shiga, Japan) and purified with protein A-agarose (Amersham Pharmacia Biotech) according to the manufacturer's protocol.

Immunoprecipitation and western blot. The COS7 green monkey kidney cell line was maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and antibiotics (A9909, Sigma). For immunoprecipitation analyses, COS7 cells at 70% confluency were cotransfected with FLAG- and HA-NTKL expression plasmids by the lipofection method using the TransIT LT-1 reagent (Panvera, Madison, WI) as described [41]. Twenty-four hours after transfection, the cells were collected and lysed in extraction buffer containing 20 mM Tris-Cl, pH 7.5, 150 mM NaCl, 0.5% Nonidet P40, and Complete protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN). After centrifugation at 20,000*g* for 5 minutes, the whole-cell extracts were subjected to immunoprecipitation using anti-FLAG monoclonal antibody M2 (Sigma), and the interaction between FLAG- and HA-tagged NTKL was analyzed by western blot as described [42].

Chemical cross-linking. MCF7 human breast cancer cell line was obtained from the Cell Resource Center for Biomedical Research, Tohoku University, and maintained as described above. Cells were lysed in phosphate-buffered saline supplemented with 0.2% Triton X-100, and the whole-cell extract was cleared by centrifugation at 20,000*g* for 5 minutes. The whole-cell extract was reacted with bis(sulfosuccinimidyl)suberate (Pierce, Rockford, IL) at increasing concentrations for 20 minutes on ice. Reactions were stopped by adding 50 mM Tris-Cl, pH 6.8, followed by incubation on ice for 15 minutes. Covalent cross-linking of the endogenous NTKL protein was analyzed by SDS-PAGE followed by western blot using anti-NTKL antibody as described above.

Immunofluorescence microscopy. MCF7 cells were seeded onto culture slides (Becton Dickinson, Bedford, MA) and transfected with the pEGFP-NTKL plasmid, as described [41]. At 1 hour after transfection, the culture medium was replaced with fresh medium. After a further incubation of 20 hours, nocodazole (Sigma) was added to the medium at 150 ng/ml to increase the fraction of mitotic cells. After 10 hours, the cells were washed twice with phosphate-buffered saline (PBS) and incubated in fresh medium for 75 minutes. The cells were fixed, permeabilized, and stained with anti-α-tubulin monoclonal antibody B512 at 15 ng/ml (Sigma) and anti-mouse antibody conjugated with Alexa 594 at 100 ng/ml (Molecular Probes, Eugene, OR) as described [41]. Stained cells were mounted in Vectashield (Vector Laboratories, Burlingame, CA) containing 4, 6-diamino-2-phenylindole (DAPI). Fluorescence was observed using an Axioplan-2 microscope (Carl Zeiss, Jena, Germany) equipped with a Quips Smart Capture System (Vysis, Downers Grove, IL).

To examine subcellular localization of endogenous NTKL protein, MCF7 and HeLa were seeded on culture slides precoated with polylysine (Biocoat, Becton Dickinson). Cells were fixed for 10 minutes at −20°C in 33% methanol/67% acetone prechilled at −20°C. The fixed cells were double-stained with the anti-NTKL and anti-α-tubulin antibodies, and subjected to fluorescence microscopy as above.

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Sequence data from this article have been deposited in the DDBJ/EMBL/GenBank Data Libraries under accession numbers AB51427 (full-length NTKL), AB051428 (variant 1), and AB047077 (variant 2).

Align two sequences

Thu Dec 9 01:38:21 "GMT 2004

```
 /usr/tmp/seq1.499457.sca :  808 aa
>SEQ ID NO:1, 808 bases, AFE764FE checksum.          808 aa vs.
>NTKL AB051427, 808 bases, 1526818B checksum.        808 aa
scoring matrix: , gap penalties: -12/-2
99.5% identity;          Global alignment score: 5480

                  10        20        30        40        50        60
/usr/t    MWFFARDPVRDFPFELIPEPPEGGLPGPWALHRGRKKATGSPVSIFVYDVKPGAEEQTQV
          ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
NTKL      MWFFARDPVRDFPFELIPEPPEGGLPGPWALHRGRKKATGSPVSIFVYDVKPGAEEQTQV
                  10        20        30        40        50        60

                  70        80        90       100       110       120
/usr/t    AKAAFKRFKTLRHPNILAYIDGLETEKCLHVVTEAVTPLGIYLKARVEAGGLKELEISWG
          ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
NTKL      AKAAFKRFKTLRHPNILAYIDGLETEKCLHVVTEAVTPLGIYLKARVEAGGLKELEISWG
                  70        80        90       100       110       120

                 130       140       150       160       170       180
/usr/t    LHQIVKALSFLVNDCSLIHNNVCMAAVFVDRAGEWKLGGLDYMYSAQGNGGGPPRKGIPE
          ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
NTKL      LHQIVKALSFLVNDCSLIHNNVCMAAVFVDRAGEWKLGGLDYMYSAQGNGGGPPRKGIPE
                 130       140       150       160       170       180

                 190       200       210       220       230       240
/usr/t    LEQYDPPELADSSGRVVREKWSADMWRLGCLIWEVFNGPLPRAAALRNPGKIPKTLAPHY
          ::::::::::::::::::::::::::::::::::::::::::::::::::::::::.:::
NTKL      LEQYDPPELADSSGRVVREKWSADMWRLGCLIWEVFNGPLPRAAALRNPGKIPKTLVPHY
                 190       200       210       220       230       240

                 250       260       270       280       290       300
/usr/t    CELVGANPKVRPNPARFLQNCRAPGGFMSNRFVETNLFLEEIQIKEPAEKQKFFQELSKS
          ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
NTKL      CELVGANPKVRPNPARFLQNCRAPGGFMSNRFVETNLFLEEIQIKEPAEKQKFFQELSKS
                 250       260       270       280       290       300

                 310       320       330       340       350       360
/usr/t    LDAFPEDFCRHKLLPQLLTAFEFGNAGAVVLTPLFKVGKFLSAEEYQQKIIPVVVKMFSS
          ::::::::::::.:::::::::::::::::::::::::::::::::::::::::::::::
NTKL      LDAFPEDFCRHKVLPQLLTAFEFGNAGAVVLTPLFKVGKFLSAEEYQQKIIPVVVKMFSS
                 310       320       330       340       350       360

                 370       380       390       400       410       420
/usr/t    TDRAMRIRLLQQMEQFIQYLDEPTVNTQIFPHVVHGFLDTNPAIREQTVKSMLLLAPKLN
          ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
NTKL      TDRAMRIRLLQQMEQFIQYLDEPTVNTQIFPHVVHGFLDTNPAIREQTVKSMLLLAPKLN
                 370       380       390       400       410       420

                 430       440       450       460       470       480
/usr/t    EANLNVELMKHFARLQAKDEQGPIRCNTTVCLGKIGSYLSASTRHRVLTSAFSRATRDPF
```

EXHIBIT C

```
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NTKL      EANLNVELMKHFARLQAKDEQGPIRCNTTVCLGKIGSYLSASTRHRVLTSAFSRATRDPF
                 430       440       450       460       470       480

                 490       500       510       520       530       540
/usr/t    APSRVAGVLGFAATHNLYSMNDCAQKILPVLCGLTVDPEKSVRDQAFKAFRSFLSKLESV
          ::::::::::::::::::::::::::::::::::::::::::::::.:::::::::::::
NTKL      APSRVAGVLGFAATHNLYSMNDCAQKILPVLCGLTVDPEKSVRDQAFKAIRSFLSKLESV
                 490       500       510       520       530       540

                 550       560       570       580       590       600
/usr/t    SEDPTQLEEVEKDVHAASSPGMGGAAASWAGWAVTGVSSLTSKLIRSHPTTAPTETNIPQ
          ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
NTKL      SEDPTQLEEVEKDVHAASSPGMGGAAASWAGWAVTGVSSLTSKLIRSHPTTAPTETNIPQ
                 550       560       570       580       590       600

                 610       620       630       640       650       660
/usr/t    RPTPEGVPAPAPTPVPATPTTSGHWETQEEDKDTAEDSSTADRWDDEDWGSLEQEAESVL
          ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
NTKL      RPTPEGVPAPAPTPVPATPTTSGHWETQEEDKDTAEDSSTADRWDDEDWGSLEQEAESVL
                 610       620       630       640       650       660

                 670       680       690       700       710       720
/usr/t    AQQDDWSTGGQVSRASQVSNSDHKSSKSPESDWSSWEAEGSWEQGWQEPSSQEPPSDGTR
          ::::::::::::::::::::::::::::::::::::::::::::::::::::::: ::::
NTKL      AQQDDWSTGGQVSRASQVSNSDHKSSKSPESDWSSWEAEGSWEQGWQEPSSQEPPPDGTR
                 670       680       690       700       710       720

                 730       740       750       760       770       780
/usr/t    LASEYNWGGPESSDKGDPFATLSARPSTQPRPDSWGEDNWEGLETDSRQVKAELARKKRE
          ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
NTKL      LASEYNWGGPESSDKGDPFATLSARPSTQPRPDSWGEDNWEGLETDSRQVKAELARKKRE
                 730       740       750       760       770       780

                 790       800
/usr/t    ERRREMEAKRAERKVAKGPMKLGARKLD
          ::::::::::::::::::::::::::::
NTKL      ERRREMEAKRAERKVAKGPMKLGARKLD
                 790       800

Elapsed time:  0:00:00
```

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Research and Development: Identification and validation of drug targets for cancer therapy, and development of anti-cancer drugs.

2002.7-2003.7 Associate director, Project leader

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Research: Validation of drug targets for inhibition of tumor cell growth or sensitization of tumor cells to the effects of chemotherapeutic agents via cell cycle regulation.

2002.1-2002.7 Group leader, Project leader

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EXHIBIT D

tumor cells to the effects of chemotherapeutic agents via cell cycle regulation.

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Research: Identification of proteins and peptides that play an important role in cell cycle regulation of specific tumor cells using retroviral functional screens.

1998.2-1998.12 Senior scientist

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Research: Characterization of a membrane receptor, Toso, which inhibit TNF receptor family-induced apoptosis.

1995.3-1998.2 Postdoctoral Fellow

Department of Molecular Pharmacology, Stanford University.

Research: Analysis of signaling pathway using high titer retrovirus.

Scientific Advisor: Assistant Professor Garry P. Nolan

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Research: Cellular mechanism of development of a retrovirus-induced immunodeficiency syndrome (MAIDS)

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1987-1991 Department of Biology,
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Research: Immunology
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Special Fellow of The Japanese Ministry of Education, Culture and Science, April 1990-March 1991.

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Publications

1. Mita, S., Harada, N., Naomi, S., **Hitoshi, Y.**, Sakamoto, K., Akagi, M., Tominaga, A. & Takatsu, K., **(1988)**. Receptors for T cell-replacing factor / Interleukin 5 Specificity, quantitation, and its implication. J. Exp. Med., 168, 863 - 878.

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Patent

1. Toso, a cell-surface specific regulator of Fas-induced apoptosis in T cells
   Stanford Docket S98-019